

Studies on Monotreme Proteins. V.* Amino Acid Sequence of the α -Chain of Haemoglobin from the Platypus, *Ornithorhynchus anatinus*

R. G. Whittaker and E. O. P. Thompson

School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033.

Abstract

Blood from the platypus contained three haemoglobins which were separated by chromatography on DEAE-Sephadex. The major component, Hb-I, was converted to globin and fractionated into the α - and β -chains by chromatography on CM-cellulose in 8M urea-thiol buffers, and the complete amino acid sequence of the 141 residues of the α -chain were determined. Peptides derived from the α -chain by tryptic digestion were isolated by paper ionophoresis and chromatography. The amino acid sequences were determined by the dansyl-Edman procedure or by further digestion with other enzymes.

The amino acid sequence showed 16 changes from the α -globin of the other extant monotreme, the echidna. There were 39 changes from the human α -globin sequence. By comparison with the 'contact sites' in horse haemoglobin there is one change in the α -haem contacts, four changes in the α_1 - β_1 contacts and no changes in the α_1 - β_2 contacts.

The date of divergence of the monotremes from the other mammals was estimated to be 180 ± 37 million years, based on the number of amino acid differences between species and allowing for multiple mutations during the evolutionary period. The significance of this estimate in relation to the time of divergence of marsupials is discussed.

Introduction

The previous papers in this series (Whittaker *et al.* 1972, 1973; Thompson *et al.* 1973; Dodgson *et al.* 1974) involved the sequencing of globin chains from the echidna, *Tachyglossus aculeatus*. The present paper is the first of a series of studies on the globin chains from the other extant monotreme, the platypus, *Ornithorhynchus anatinus* (Shaw, 1799). In this paper the sequence of the α -chain from the major haemoglobin of the platypus is reported.

The monotremes present a problem to palaeontologists as no fossil remains older than 10 million years (Young 1962) are evident. The lack of teeth in adult forms adds to the problem as a large amount of palaeontological classification is based on this feature. This lack of fossil evidence led to a number of proposals concerning the origins and affinities of the monotremes as compared with the other mammalian groups. The commonly accepted view (Simpson; see Hopson and Crompton 1969) is that the mammals as a group have a polyphyletic origin with the monotremes crossing the 'mammalian threshold' independently of the other mammals by parallel evolution from ancestral mammal-like reptiles in the late Permian, about 240 million years ago. Hopson and Crompton (1969) propose that the mammals have a monophyletic origin, having radiated from a single ancestral species in the late

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Triassic (about 190 million years ago). On the other hand, Gregory (see Griffiths 1968) proposed that the monotremes were more closely related to the marsupials than to the placental mammals, thus implying a much later date for the divergence of the monotremes. Comparison of the sequences of globin chains (Zuckerklund and Pauling 1962) could help to resolve the questions regarding the date of divergence of the monotremes.

Materials and Methods

The methods of high-voltage ionophoresis, peptide mapping, amino acid analysis, cyanogen bromide cleavage, sequence determination by the dansyl-Edman procedure and digestion with trypsin, chymotrypsin and thermolysin were the same as previously described (Air and Thompson 1969, 1971; Beard and Thompson 1971; Whittaker *et al.* 1972).

Fractionation of Haemolysate

The blood sample was from an animal collected in the Snowy Mountains area of southern New South Wales. The samples were freed from plasma by centrifugation, and the red cells were washed with isotonic saline before being stored in 40% ethylene glycol containing 6% trisodium citrate at -20°C (cf. Cooper *et al.* 1973). Prior to haemolysis the stored cells were washed with isotonic saline. The cells were then haemolysed as described by Thompson *et al.* (1969). The cell debris was washed once with two volumes of water and the combined solutions converted into the carbon monoxide forms. Starch gel electrophoresis of the haemolysate gave a strong major haemoglobin band and a more diffuse minor component.

The haemolysate was fractionated on a column of DEAE-Sephadex (2.8 by 26 cm) equilibrated with 0.05M tris-HCl (pH 8.2) containing 100 mg KCN per litre (Dozy *et al.* 1968) and developed with a similar strength buffer with the pH gradually decreasing to 7.5 (linear gradient device with 500 ml/chamber). The column was further developed in a stepwise manner (500 ml of pH 7.5 buffer followed by 500 ml of pH 7.3 buffer and finally by 500 ml of pH 6.7 buffer). The fractionation yielded three components designated Hb-I, Hb-II and Hb-III in order of elution (Fig. 1*a*). The approximate proportion of each fraction was 80% Hb-I, 15% Hb-II and 5% Hb-III. On cellulose acetate electrophoresis at pH 8.6 (Pabis *et al.* 1968), Hb-I and Hb-III appeared pure while Hb-II was contaminated with Hb-I.

Fractionation of Globin-I

The globin was prepared from Hb-I and samples were chromatographed on a CM-cellulose column (1.8 by 15 cm) according to the method of Clegg *et al.* (1965), using a linear gradient from 300 ml each of 0.012M Na^+ (phosphate) and 0.037M Na^+ (phosphate) in 8M urea containing 0.05M mercaptoethanol (Fig. 1*b*). The column was equilibrated at the lower salt concentration. The separated globin chains were carboxymethylated before recovery by dialysis and freeze-drying (Thompson *et al.* 1969).

Citraconylation of the α -Chain from Globin-I

The α -chain was citraconylated using a slight variation of the procedure of Gibbons and Perham (1970). The buffer strength was doubled to 0.1M *N*-ethylmorpholine and the reaction carried out at room temperature. After tryptic digestion the material soluble in the starting buffer was chromatographed on a Cellex-D column (1.8 by 14 cm), using a linear gradient of 0.5 \rightarrow 5% ammonium bicarbonate, pH 8.0 (250 ml/chamber).

Isolation of Tryptic Peptides

After tryptic digestion of the α -globin at pH 8.7 the digestion mixture was freeze-dried and pyridine-acetate buffer (pH 6.4) was added. The material soluble at pH 6.4 was peptide-mapped (Thompson *et al.* 1969).

The material insoluble at pH 6.4 was freeze-dried and 3.5% formic acid (pH 1.9) was added. The soluble material was fractionated by ionophoresis at pH 1.9 and the small amount of insoluble material was washed once with the pH 1.9 buffer and the residue taken for sequence analysis.

Estimation of Dates of Divergence

The α -chain sequence of platypus plus the two previously reported α -chain sequences for the echidna (Thompson *et al.* 1973; Whittaker *et al.* 1973) were used to estimate a date of divergence of the monotremes from the other mammalian groups. The method was based on that of Air *et al.* (1971) and Thompson and Air (1971) with the following modifications.

1. The phylogenetic tree derived from palaeontological evidence was altered to accommodate, where possible, the dates suggested by Romero-Herrera *et al.* (1973). The older alternative for the marsupial-placental divergence was used.
2. The number of α -chain sequences used to calculate the rate was the same as that used by Air *et al.* (1971), but the dog α -chain sequence was substituted for the monkey sequence. The monkey α -chain sequence was deleted to avoid undue emphasis of the primate lineage rate.
3. To allow for hidden mutations the accepted point mutation (PAM) units of Dayhoff (1972) were utilized.

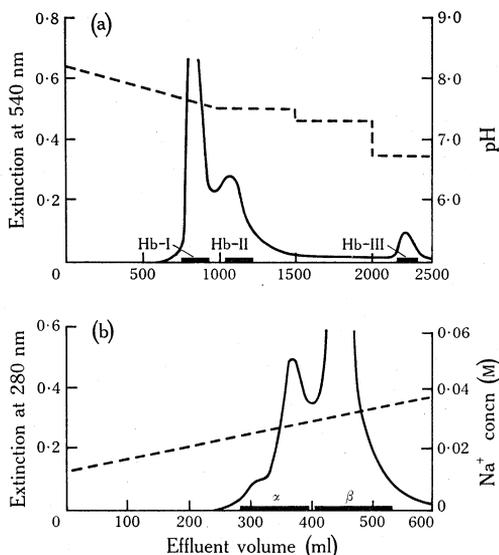


Fig. 1. (a) Separation of platypus haemoglobins (carbon monoxide forms) on a DEAE-Sephadex column (2.8 by 26 cm). The haemoglobins were eluted with 0.05M tris-HCl containing 100 mg KCN/l, by a combination of linear pH gradient and stepwise pH elution as shown by the dashed line. The fractions pooled are indicated by bars and the major fraction, designated Hb-I, was taken for further investigations. (b) Separation of globins from platypus haemoglobin Hb-I using a linear concentration gradient (---) on a CM-cellulose column (1.8 by 15 cm) in 8M urea-thiol buffers. Initial buffer 8M urea-0.05M mercaptoethanol-0.012M Na⁺ (phosphate), pH 6.8 (300 ml), limit buffer 8M urea-0.05M mercaptoethanol-0.037M Na⁺ (phosphate), pH 6.8 (300 ml), flow rate 55 ml/h. Approximately 150 mg of globin was loaded in each case. The fractions pooled for sequence studies are indicated by bars. The shoulder on the α -globin peak is a carbamoylated fraction.

Results

As in previous papers, tryptic peptides are numbered from the *N*-terminus following the nomenclature for human α -chain (Gerald and Ingram 1961). The symbols A and B have been added where an extra lysine or arginine residue has occurred in the platypus chain. When a lysine residue has not occurred in the platypus chain the peptide has been given all the numbers of the corresponding human peptides (see Fig. 2).

Fractionation of Tryptic Peptides

The peptide map for the whole digest obtained by paper ionophoresis–chromatography is shown in Fig. 3. All tryptic peptides except α Tp12A occurred in the peptide map. α Tp4 was further purified from α Tp1 by ionophoresis at pH 1.9. α Tp6 was isolated in a low yield, being largely insoluble at pH 6.4.

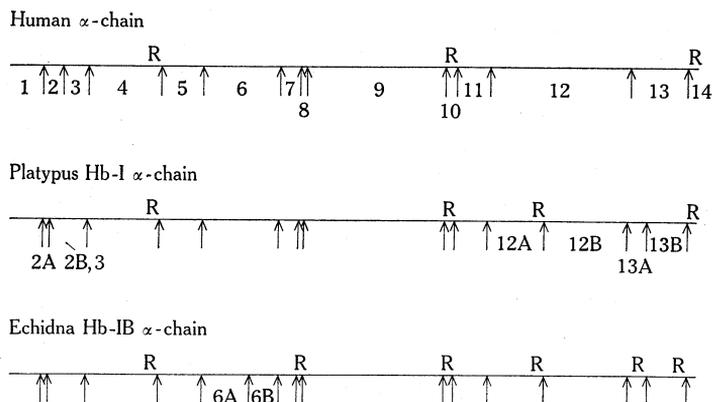


Fig. 2. Nomenclature for α -chain tryptic peptides of the major haemoglobin of the platypus compared with α -chain tryptic peptides of human and echidna-IB haemoglobins. The distribution of lysine and arginine residues in echidna α -chain is shown to indicate the strong similarities between both monotremes. The diagrams are scaled to show the relative number of residues in each peptide. \uparrow Lysine residue. $^R\uparrow$ Arginine residue.

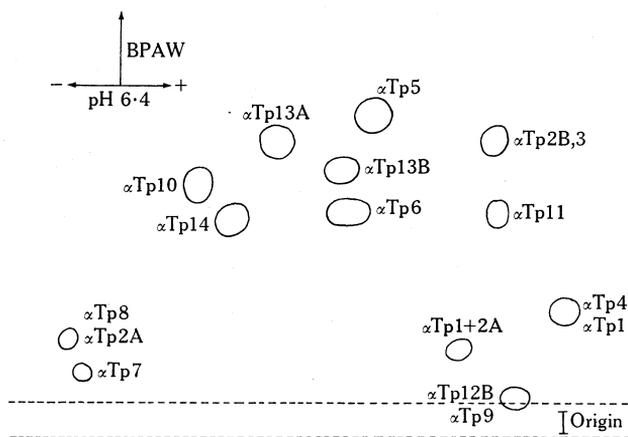


Fig. 3. Peptide map of the tryptic peptides of the α -chain of platypus globin. Ionophoresis at pH 6.4 was followed by chromatography with butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v; BPAW) as indicated. The peptides are given the identification number corresponding to their position in the chain as shown in Fig. 2.

Isolation of α Tp12B following Citraconylation

From the amino acid composition (Table 1) platypus α -globin contained four arginyl residues so that tryptic digestion of the citraconylated α -chain would be

expected to produce five peptides. However, as the C-terminal residue was arginine (invariant in all sequenced α -chains) four citraconyl peptides were produced. Separation of the tryptic peptides of citraconylated α -chain on a Cellex-D column gave two major fractions: the first fraction eluted contained α Cit4 and the second α Cit1 and α Cit2. α Cit3 was not eluted from the column and was presumably present in the insoluble fraction (the fractions were numbered in order from the N-terminal).

A pure sample of α Tp12B was isolated from a tryptic digest of α Cit4 after decitraconylation.

Table 1. Amino acid composition of platypus α -globin

Samples were hydrolysed at 110–115°C for 24 h under vacuum with 6N HCl containing 1 mg/ml phenol. Values are given as moles per mole of protein and are uncorrected for losses during hydrolysis or due to incomplete hydrolysis. Also shown is the amino acid composition determined by sequence data

Amino acid	Platypus α -globin:		Amino acid	Platypus α -globin:	
	Hydrolysate	Sequence		Hydrolysate	Sequence
Lysine	12.5	12	Glycine	8.3	8
Histidine	9.8	10	Alanine	20.2 ^A	22
Arginine	4.2	4	Valine	8.1	8
SCM-cysteine	1.6	2	Methionine	2.2	3
Aspartic acid	10.1	10	Isoleucine	1.9	2
Threonine	7.2	9	Leucine	16.0	16
Serine	7.5	10	Tyrosine	2.9	3
Glutamic acid	9.6	9	Phenylalanine	7.6	8
Proline	4.1	4	Tryptophan	1.1 ^B	1

^A The initial composition was determined at the start of sequencing. At the completion of sequencing all values are in agreement with the composition determined by sequence except for the alanine values. A second sample was hydrolysed at approximately 120°C for 24 h and gave 22.3 alanine with respect to 16 leucine residues.

^B Determined by the spectrophotometric method of Beavan and Holiday (1952).

Isolation of α Tp6 and α Tp12A

α Tp6 and α Tp12A were isolated from the material insoluble at pH 6.4 in the original tryptic digest. Ionophoresis of this material at pH 1.9 gave a rapidly migrating peptide which on analysis and sequencing was found to be α Tp6. α Tp12A was partially soluble at pH 1.9, being found at the origin and streaking away from the origin on the pH 1.9 ionopherogram. The insoluble material at pH 1.9 also proved to be α Tp12A on sequencing. The peptide at the origin was the purest sample of α Tp12A on analysis.

Amino Acid Compositions and Sequences of Tryptic Peptides

The amino acid compositions of purified peptides are shown in Table 2. The total compositions are in good agreement with the total analysis of the α -chain (Table 1).

In the sequences, all residues identified by the dansyl-Edman method are printed in *italic* font. The alignment of the tryptic peptides has been based on homology with previously sequenced α -chains. Confirmation of this alignment, to a certain extent, has been given by the results of cyanogen bromide cleavage, chymotryptic cleavage and the tryptic peptides obtained following citraconylation.

Table 2. Amino acid composition of platypus α -globin tryptic peptides

Soluble peptides were purified by paper ionophoresis at pH 6.4 and paper chromatography. Hydrolysis was at 110°C for 24 h. Values are not corrected for losses during hydrolysis or due to incomplete hydrolysis and are given as moles per mole of peptide, with preferred values in parentheses. Detection by ninhydrin results in low recovery of the N-terminal amino acid residues

Amino acid	α Trp1 + 2A ^A	α Trp2B,3	α Trp4	α Trp5	α Trp6	α Trp7	α Trp8	α Trp9 ^B	α Trp10	α Trp11	α Trp12A	α Trp12B ^C	α Trp13A	α Trp13B	α Trp14	Total in protein
Lys	2.0(2)	1.0(1)	0.8(1)	1.0(1)	1.1(1)	1.0(1)	+ ve(1)	0.9(1)	1.0(1)	1.0(1)	0.2	0.8(1)	1.0(1)	1.1(1)		12
His			0.8(1)		1.9(2)	0.9(1)		2.4(3)			1.0(1)	1.2(2)				10
Arg			0.9(1)						1.0(1)		0.8(1)				1.0(1)	4
SCM/Cys																
Asp	1.0(1)				1.0(1)			4.9(5)			0.3(1)	0.4(1)				2
Thr	0.8(1)	0.8(1)		1.5(2)	0.8(1)			1.0(1)		2.0(2)	0.5	1.1(1)				10
Ser					2.6(3)			3.2(4)			0.3	1.0(1)		1.7(2)		9
Glu			4.1(4)	1.0(1)	1.2(1)						0.5	1.0(1)	0.9(1)	1.0(1)		10
Pro	1.0(1)	0.8(1)		0.8(1)	1.2(1)					1.0(1)	0.4	1.0(1)				9
Gly		1.0(1)	3.2(3)		1.3(1)	1.1(1)		1.2(1)			+	1.9(2)				4
Ala	1.1(1)	1.0(1)	3.9(4)	1.1(1)	1.1(1)	1.0(1)		7.0(7)			2.4(2)	2.9(3)		1.0(1)		22
Val		0.9(1)						1.0(1)		2.0(2)	1.8(2)			1.6(2)		8
Mes ^D	+ ve(1)							+ ve(1)				+ ve(1)				3
Ile					0.9(1)						0.9(1)					2
Leu	1.0(1)	0.8(1)	1.0(1)	0.6(1)	0.9(1)			3.6(4)	0.8(1)		3.0(4)		1.0(1)	0.9(1)		16
Tyr			0.7(1)		0.5(1)						+				0.8(1)	3
Phe				1.8(2)	1.9(2)			1.0(1)		1.0(1)	0.5	0.9(1)	0.8(1)			8
Trp			+ ve(1) ^E													1
Total	8	8	15	9	16	4	1	29	2	7	12	16	4	8	2	141

^A This overlapping peptide was the major product of tryptic digestion.

^B Corrected for 40% contamination by α Trp12B.

^C Purified from a tryptic digest of α Cit4 after deacetylation.

^D Methionine sulphone.

^E Detected by the Ehrlich reagent.

αTp12B

The sequence of this peptide was

His-SCMCys-Pro-Gly-Glu-Phe-Thr-Pro-Ser-Ala-His-Ala-Ala-Met-Asp-Lys.
112127

Direct degradation gave a similar overlapping effect as encountered with the sequencing of echidna *αTp12B* (Whittaker *et al.* 1973). At all positions other than the *N*-terminal one, two dansylamino acids occurred, the minor dansylamino acid (approx. 25%) being from the following residue. A possible reason for this behaviour, suggested by Thomsen *et al.* (1972), is the use of a volatile buffer with limited buffering capacity in the Edman degradation.

The direct degradation of *αTp12B*, interpreted after allowing for this overlap, gave X-SCMCys-Pro-Gly-Glx-Phe-Thr-Pro-Ser. Owing to the destruction of Dns-SCMCys on acid hydrolysis, Dns-Pro appeared to be the major dansylamino acid at position 113 with Dns-SCMCys the minor. However, Dns-Pro was the major derivative at position 114 with Dns-Gly the minor.

From a cyanogen bromide cleavage of intact *α*-chain, two peptides from *αTp12B* were isolated. The compositions of these peptides were consistent with the presence of a methionine residue at position 125. The *C*-terminal peptide was Asp-Lys, which was neutral at pH 6.4, indicating an aspartic acid residue.

From a thermolysin digest of the *N*-terminal peptide fragment three peptides were isolated and analysed for amino acid composition and *N*-terminal residue. The peptides were

Th1 *Phe*-Thr-Pro-Ser
Th2 *Ala*-His
Th3 *Ala*-Ala-Hse

A peptide corresponding to the *N*-terminal sequence of *αTp12B* was not detected with ninhydrin.

Chymotryptic digestion of intact *S*-carboxymethylated *α*-chain gave a peptide which analysed as His_{0.8}Arg_{0.9}Cysteic acid_{0.6}Glx_{1.1}Pro_{0.9}Gly_{1.0}Ala_{0.9}Phe_{1.0}. (Hydrolysis of the peptide was not carried out *in vacuo*, which explains the detection of cysteic acid.) This provided an overlap of the *C*-terminal Ala-Arg of *αTp12A* with the *N*-terminal sequence of *αTp12B* to residue 117. This peptide was neutral at pH 6.4, indicating the presence of a glutamic acid residue. A histidine residue can be assigned to the *N*-terminal position of *αTp12B* by difference.

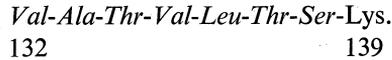
From the original tryptic digest the slightly basic peptide *Ala-Ala-Met-Asp-Lys* was isolated. The peptide was apparently formed due to the presence of contaminating chymotrypsin in the trypsin preparation which resulted in the cleavage of the His-Ala peptide bond of *αTp12B*. The slight basicity of the peptide was probably due to incomplete ionization of the aspartic acid residue due to the proximity of the lysine residue.

αTp13A

This peptide sequenced directly as *Phe-Leu-Ser-Lys*.
128131

α TP13B

Direct determination gave the sequence as



Dns-Val-Leu was a major product at residue position 135.

 α TP14

Direct sequencing gave *Tyr-Arg.*

140 141

Complete Sequence

The complete amino acid sequence of the platypus α -chain is shown in Table 3.

Table 3. Complete amino acid sequence of the α -chain of platypus haemoglobin

Residues are numbered from the *N*-terminal residue and the positions of cleavage by trypsin are shown by arrows. The dashed arrow indicates a site of incomplete cleavage. The 16 residues that differ from those present in echidna Hb-IB α -chain are in capital letters

5	10	15	20
MET-Leu-Thr-Asp-Ala-Glu-Lys-Lys-Glu-Val-Thr-ALA-Leu-Trp-Gly-Lys-Ala-ALA-Gly-His-GLY-			
α Tp1	\uparrow α Tp2A	α Tp2B,3	\uparrow
25	30	35	40
Glu-Glu-Tyr-Gly-Ala-Glu-Ala-Leu-Glu-Arg-Leu-Phe-GLN-ALA-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-			
α Tp4	\uparrow	α Tp5	\uparrow
45	50	55	60
Phe-Ser-His-PHE-Asp-Leu-Ser-HIS-Gly-Ser-Ala-Gln-ILE-Lys-Ala-His-Gly-Lys-LYS-Val-Ala-Asp-			
α Tp6	\uparrow	α Tp7	\uparrow α Tp8 \uparrow
65	70	75	80
Ala-Leu-SER-Thr-Ala-Ala-Gly-His-Phe-ASP-Asp-Met-Asp-Ser-Ala-Leu-Ser-Ala-Leu-Ser-Asp-Leu-			
α Tp9			
90	95	100	105
His-Ala-His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ala-His-Cys-ILE-Leu-Val-Val-			
\uparrow α Tp10 \uparrow	α Tp11	\uparrow	α Tp12A
110	115	120	125
Leu-Ala-Arg-His-CYS-Pro-GLY-Glu-Phe-Thr-Pro-Ser-Ala-His-Ala-Ala-Met-Asp-Lys-Phe-Leu-			
\uparrow	α Tp12B		\uparrow α Tp13A
130	135	140	
Ser-LYS-Val-Ala-Thr-Val-Leu-Thr-Ser-Lys-Tyr-Arg			
\uparrow	α Tp13B	\uparrow α Tp14	

Estimation of the Monotreme Date of Divergence

The α -chain sequences compared are shown in Table 4. The chains were scanned using the computer method of Beard (1971) to give the number of amino acid differences between chains. These data were then used to determine PAM units (number

of accepted point mutations per 100 links of two sequences) from the information given by Dayhoff (1972). By use of the PAM units shown in Table 4 (omitting the monotreme comparisons) and the dates of divergence estimates (Table 5) the average time for one accepted point mutation to occur per 100 residues of one chain was calculated to be 8.87 ± 0.85 million years (95% confidence interval for the mean, $n = 28$).

Table 4. Matrices of differences between α -globins

The matrix of observed amino acid differences between α -chain sequences compared over 143 residues is shown in the upper triangle. PAM units (per 100 links) are shown in the lower triangle

	Human	Rabbit	Dog	Horse	Bovine	Kangaroo	Chicken	Carp	Echidna 1 ^A	Echidna 2 ^B	Platypus
Human		25	23	18	17	27	35	71	37	41	39
Rabbit	20		27	25	25	37	43	74	48	49	46
Dog	18	22		27	28	33	44	70	42	42	45
Horse	14	20	22		18	29	40	70	42	42	42
Bovine	14	20	23	14		26	38	68	43	44	44
Kangaroo	22	33	28	23	21		41	74	49	49	46
Chicken	31	39	40	36	34	38		75	48	51	47
Carp	83	88	81	81	77	88	90		78	76	77
Echidna 1 ^A	33	46	38	38	39	47	46	96		9	16
Echidna 2 ^B	37	47	38	38	40	47	50	92	7		22
Platypus	35	43	42	39	41	43	45	94	13	18	

^A Echidna α -chain from Hb-IB.

^B Echidna α -chain from Hb-IIA.

Table 5. Palaeontological estimates of the dates of divergence of the species

Divergence point	Estimated time of divergence (million years)
Carp-chicken, mammals	350 ^A
Chicken-mammals	220 ^A
Marsupials-placental mammals	104 ^B
General radiation of placental mammals	68 ^B
Horse-bovine	55 ^B

^A Air *et al.* (1971).

^B Romero-Herrera *et al.* (1973).

By application of the mean to the PAM units obtained by comparing the monotreme sequences with all the other mammalian sequences a range of estimates for the date of divergence of the monotremes was obtained. The mean of this range was 180 million years with a 95% confidence interval of 143–217 million years ($n = 18$). Similarly, the date of the marsupial-placental divergence was estimated at 113 million years. This variation from the estimate of Air *et al.* (1971) (139 million years) was caused mainly by the use of the amended divergence dates estimated by palaeontological methods.

The calculation for the platypus and echidna divergence point gave an average estimate of 67 million years.

Discussion

The length of the α -globin peptide of the platypus is 141 residues, identical with all previously sequenced α -chains with the exception of carp.

The distribution of trypsin-sensitive basic amino acid residues is similar to the distribution in echidna α -chains with the exception that an arginine residue at position 50 is replaced by a histidine residue; histidine commonly occurs in this position. The occurrence of an arginine residue at position 111, as in the echidna, results in a fragmentation of the region of the α -chain usually associated with the tryptic 'core'. Arginine, while not occupying this position in any other α -chain, commonly occurs in the corresponding position in β -chain sequences.

On comparison with all the previously sequenced α -globins, five changes have occurred that are not listed by Dayhoff (1972). Position 1, usually occupied by an invariant valine residue, is methionine in platypus. This was also the *N*-terminal residue in Hb-II of chicken (Moss and Thompson 1969). This change could arise through a one-point mutation. It is the only terminal residue clearly visible in the intact haemoglobin molecule, and it is not thought to be involved in binding between like chains (Perutz 1969). Positions 35 (Ala) and 67 (Ser) could also arise by one-point mutations and the changes appear conservative; position 35 is usually serine and 67 is usually threonine. Position 131 (Lys) is an extremely variant position usually occupied by hydrophilic uncharged residues. A basic residue, arginine, also occurs in this position in the echidna. The most striking change is the occurrence of cysteine at position 113, a site that is normally occupied by either leucine or histidine. At least two point mutations from the codons for histidine or leucine are required to give the cysteine codon. The effect of a much smaller side-chain on the structure is not known and it may be insignificant as, according to Perutz *et al.* (1965), this position occurs at the end of a helical section and can be occupied by either polar or non-polar residues. All five changes probably have little effect on the overall structure.

Compared to horse α -chain there have been few changes from the functional point of view in 'contact sites' (Perutz 1969). Of the 19 contact sites between α -chain residues and the haem group, 18 are identical with horse. The change of leucine for methionine at position 32 (B13) has previously been reported for the echidna α -chain sequence. The residues involved in α_1 - β_2 contacts are identical to those in horse α -chain. The α_1 - β_1 contacts show four changes; three of these have been reported in other α -chain sequences and are glutamine at position 34 (leucine in horse), valine at position 107 (serine), and arginine at position 111 (valine). The remaining variation is an alanine residue at position 35 which in horse is occupied by glycine and is serine in most other species. While this change has not previously been observed it is unlikely to make any significant structural alteration to the molecule.

The calculation of a date of divergence of the monotremes from the other mammalian groups using the sequence of platypus α -chain and the sequences of echidna α -chains previously reported (Whittaker *et al.* 1973; Thompson *et al.* 1973) gives an estimate of 180 ± 37 million years. This estimate indicates a point of divergence from the main stream of mammalian development in the Triassic and supports the monophyletic origin theory of Hopson and Crompton (1969). The theory of Gregory (see Griffiths 1968) that the monotremes are more closely related to the marsupials is not supported. While the estimated date supports Hopson and Crompton

(1969), the polyphyletic origin theory of Simpson cannot be discounted due to the inaccuracies inherent in the dates of divergence estimated by palaeontological methods. The necessary application of the mean rate of mutation to the monotreme lineage must also be questioned as a number of workers have found that different lineages mutate at varying rates (Goodman *et al.* 1971; Dayhoff 1972; Romero-Herrera *et al.* 1973).

Intraspecies variation in mutation rates also occur as exemplified by the echidna. Usually the mutation rate of the β -chain is faster than that of the α -chain (Air *et al.* 1971), whereas in the echidna it is less. Calculations based on the echidna β -chain sequence would give a date of divergence estimate different from that given by α -chain and, in fact, more recent than the divergence point of the marsupials. This slower rate of mutation of the β -chain has previously been observed in comparisons between the globin chains of the dog and rabbit.

The β -chain of platypus haemoglobin is presently being sequenced and it will be interesting to see what pattern emerges.

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